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Could Plant Hormones Provide a Reliable Tool for Early Detection of *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae) Infested Palms?

Óscar DEMBILIO[1]* Blas AGUT[2] Maria Victoria IBÁÑEZ-GUAL[3]
Victor FLORS[4] Josep Anton JAQUES[5]

[1,5]Universitat Jaume I (UJI), Unitat Associada d'Entomologia
Agrícola UJI-IVIA, Departament de Ciències Agràries i del Medi
Natural, Campus del Riu Sec, E-12071, Castelló de la Plana, SPAIN
[2,4]UJI, Metabolic Integration and Cell Signalling Group, Departament de Ciències
Agràries i del Medi Natural, Campus del Riu Sec, E-12071, Castelló de la Plana,SPAIN
[3]UJI, Departament de Matemàtiques, Institute of Mathematics and
Applications, Campus del Riu Sec, E-12071, Castelló de la Plana, SPAIN
*Present address: TRAGSA, Centre d'Evolució d'Insectes Estèrils, Institut Valencià
d'Investigacions Agràries, Ctra. Montcada-Nàquera km 4,5; E-46113-Montcada, SPAIN
e-mail: *odembili@uji.es, bagut@uji.es, mibanez@uji.es, flors@uji.es, josep.jaques@uji.es
ORCID IDs: [1]0000-0002-9052-9121, [3]0000-0001-7043-8233, [4]0000-0003-3974-9652,
[5]0000-0003-1353-1727

ABSTRACT

Rhynchophorus ferrugineus (Olivier) (Coleoptera: Curculionidae) is one of the most important pest of palms worldwide. A serious management problem is the difficulty of detecting early infestation stages, which is critical for successful control. Our main objective has been to characterize the metabolic response of *Phoenix canariensis* hort. ex Chabaud to *R. ferrugineus* injury to identify candidate biomarkers for early detection. Mechanical wounding and *R. ferrugineus* infestation resulted in different patterns of plant hormone and secondary metabolite production: SA and caffeic acid concentrations increased by several orders of magnitude following *R. ferrugineus* development within the palm 7 days after infestation. These compounds did not change in mechanically wounded palms. Therefore, these substances could be further exploited as early warning signs of infestation.

Key words: Red palm weevil, Canary islands date palm, phythormones, plant response.

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INTRODUCTION

The red palm weevil, *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae), is a palm borer native to South Asia. Due to the unintended movement of infested material, this species has nowadays a broad distribution covering almost all palm growing areas worldwide (EPPO, 2008, 2009; Rugman-Jones, Hoddle, Hoddle & Stouthamer, 2013; Dembilio & Jaques, 2015; Milosavljević et al, 2018). As a result, this weevil has become one of the most destructive palm pests and is threatening palms worldwide (Dembilio, Riba, Gamón, & Jacas, 2014; Dembilio & Jaques, 2015; Jaques et al, 2017). In the European Union, *R. ferrugineus* is the major pest of palms, mostly *Phoenix canariensis* hort. ex Chabaud, an endemic palm to the Canary Islands, commonly used as ornamental in the northern shores of the Mediterranean basin and elsewhere (Dembilio & Jacas, 2011). Plant damage starts when adult females lay their eggs at the base of the fronds in holes made with their rostra. Neonate larvae bore into the palm core making channels and feeding on its inner contents. As larvae molt, grubs progressively tend to feed on the soft tissues surrounding the apical meristem until they complete development. Then, mature larvae migrate to the periphery of the stem to pupate. This cycle can take from 40 to 160 days depending on temperature and palm species (Dembilio & Jacas, 2011). A new generation emerges and these adults may remain within the same palm and reproduce until the apical meristem is destroyed resulting in the palm death (Dembilio & Jacas, 2011).

Due to these cryptic habits, a serious problem for the management of *R. ferrugineus* is the difficulty of detecting the early stages of infestation (Dembilio & Jaques, 2015; Dembilio, Jacas, & Llácer, 2009) which is key for its successful control. Different approaches have been explored so far including visual, acoustic, thermal, and olfactory sensing (Dembilio & Jaques, 2015; Jaques et al, 2017). However, their success is limited and nowadays visual detection is the most commonly used system. Yet, when first visual symptoms are detected it is often too late for the palm to recover (Dembilio & Jacas, 2011). Identifying how palms respond and deal with defense activation upon *R. ferrugineus* attack remains poorly known (Cangelosi et al, 2015; Giovino et al, 2015; Rasool et al, 2015) although this information could lead to novel approaches for early detection of this pest.

Arthropod herbivory activates in plants different responses. Once the plant has identified the attack, it can respond through activation of diverse defense genes controlled by phytohormones such as abscisic, salicylic and jasmonic acids (ABA, SA and JA, respectively) and ethylene (ET) (Glazebrook, 2001; Flors, Ton, Jakab & Mauch-Mani, 2005; Erb, Meldau, & Howe*,* 2012; Agut, Gamir, Jacas, Hurtado, & Flors, 2014). Indeed, Giovino et al (2015) identified key *R. ferrugineus*-modulated genes involved in *P. canariensis* innate response belonging to auxin, JA and SA pathways. These activations can result in the production of antibiotic and antixenotic compounds that can exert a negative effect on the herbivore fitness (Bennett & Wallsgrove, 1994; Chen, Gonzales-Vigil, Wilkerson, & Howe, (2007). Cangelosi et al (2015), for instance, identified filiferol, a chalconoid analogue which may be involved in antibiosis to *R. ferrugineus* in *Washingtonia filifera* Wendl. (Dembilio et al, 2009). Similarly, Rasool et al (2015) identified several differentially accumulated peptides in infested date palms, *Phoenix dactylifera* L. In the case of *R. ferrugineus* infested *P. canariensis*, Giovino et al (2015) identified some upregulated genes involved in the biosynthesis of some S-containing compounds with well-known defensive properties. However, this activation ocurred too late to promptly counteract *R. ferrugineus* attack. Interestingly, though, these compounds and those implicated in their synthesis activation could become a precious tool to detect *R. ferrugineus* feeding/oviposition activity. If they could be unambiguously identified early enough, they could allow for a timely management of this pest.

Therefore, the main objective of the present study has been to characterize the metabolic response of *P. canariensis* to early *R. ferrugineus* injuries relative to control and mechanically injured plants, with the purpose of identifying candidate biomarkers for *R. ferrugineus* early detection.

MATERIAL AND METHODS

Plant material

Commercial pesticide-free healthy 7-year-old potted *P. canariensis* palms were used. The stipe of these palms was around 40 cm high and 35 cm wide. They were planted in 50-L containers and were watered every other day. These palms were enclosed together in groups of four in nine separate cages (36 palms in total) in a mesh house starting two months prior to the onset of the assay. This time was fixed to ensure that any possible previous infestation would be detected beforehand.

Experimental insects

Adult weevils collected in the province of Valencia in traps baited with ferrugineol (*R. ferrugineus* aggregation pheromone), ethyl acetate and pieces of palm fronds were used to directly infest palms.

Infestation

Palms in six out of the nine cages included in this assay were infested at the beginning of the assay (day 0) by releasing four adult weevils (three females and one male) per palm for one week. The remaining three cages constituted the control group. On day 0, different pinnae from the 12 control palms were removed with scissors. In total 100 g per palm were collected (time 0). Likewise, a second sample was obtained from the same palms seven days later (i.e., 7 days mechanically injured palms treatment). On that day, a similar sample was obtained from 12 palms enclosed in three of the *R. ferrugineus* infested cages (i.e., 7 days post infestation, dpi, treatment). One week later, the nine *R. ferrugineus*-infested palms in the remaining three cages were similarly sampled (i.e., 14 dpi treatment). At this time, all palms were dissected to assess their infestation status. In all cases, pinnae samples were frozen immediately after removal at -80ºC for further processing.

Sample processing

Frozen samples were first ground using a refrigerated crusher and lyophilized. Subsequently, a mixture of internal standards containing d6ABA, d4SA, d6IAA and dhJA at 100 mg kg^{-1} each was added to each sample. Dry tissue (0.05 g) was homogenized to a fine powder. Subsequently 2 ml of extraction solution (H2O:MeOH 90:10 containing 0.01% HCOOH) was added to 50 mg of frozen dried samples. After polytron homogenization on ice, samples were centrifuged for 35 min at 4.000 × g at 4ºC and the supernatant was recovered and adjusted to pH 2.8 with 6 % acetic acid and subsequently partitioned twice against an equal volume of diethyl ether and centrifuged for 3 min at 4.000 × g. Then, the organic phases were combined and evaporated using a Speed-Vac (Eppendorf®) at room temperature. The solid residue was re-suspended in 1 ml of a methanol/water (10:90, vol:vol) solution and filtered through a 0.22-lm cellulose acetate filter (13 mm pk/100 TR-200430; Olimpeak Teknokroma, Barcelona, Spain). A 20-ul aliquot of this solution was then directly injected into the HPLC system. Analyses were carried out using a Waters Alliance 2690 HPLC system (Waters®) with a Kromasil reversed phase column (100 2 mm i.d.; 5 lm; Scharlabl®). The chromatographic system was interfaced with a Quatro LC (quadrupole-hexapole-quadrupole) mass spectrometer (Micromass®). MASSLYNX NT software version 4.1 (Micromass®) was used to process the quantitative data from calibration standards and the plant samples. The calibration curves were obtained by following the protocol of Flors, Ton, Van Doorn, Jakab, García-Agustín, & Mauch-Mani (2008) and Durgbanshi et al (2005).

Statistical analyses

Hormone and secondary metabolite concentrations in control (time 0) and 7-day mechanically injured palms were compared using a dependent *t*-test for paired samples. Subsequently, both treatments were separately compared to 7 and 14-dpi treatments using 1-way ANOVA. When necessary, Tukey post-hoc test was used to separate means. In the case of JA-isoleucine (JA-Ile), as this compound remained undetectable in control palms, concentrations in infested plants were compared using a *t*-test.

RESULTS

No differences for the concentrations of SA, Indole-3-acetic acid (IAA), Caffeic, Ferulic, Chlorogenic and Cinnamic acids were found between control and mechanically injured palms (Tables 1, 2). However, when mean concentrations of both treatments were compared to infested palms, significant differences were observed in all cases except for chlorogenic acid (Tables 1, 2). Cinnamic acid became undetectable (< 5 10^{-3} µg/ l) in infested palms. Contrarily, caffeic acid concentration increased more than 10-fold in infested palms within the time frame considered (14 days). SA also increased in infested palms and there were significant differences between dates with a 5- and 8-fold increase relative to control 7 and 14 dpi, respectively. Both IAA and ferulic acid concentrations increased 7 dpi but decreased seven days later and in the case of ferulic, differences between mechanically injured palms and those infested even disappeared 14 dpi.

ABA and ABA-glucoside (ABA Glu) concentrations were higher in mechanically injured palms relative to control ones (Tables 1, 2). These concentrations were even higher in *R. ferrugineus* infested palms (about 3 times) and no differences between 7 and 14 dpi were observed. Contrarily, Glucosyl Salicylate (SAG) and JA were higher in control relative to mechanically injured palms and their concentrations were even higher in infested plants, especially SAG which presented a 20-fold increase 14 dpi compared to control. Finally, JA-Ile, the active form of JA, which was not detectable in control palms, could be detected in both mechanically injured and infested palms, especially 14 dpi.

Palms used at 7 and 14 dpi had a mean of 12.08 ± 0.80 and 11.17 ± 0.54 (max. 16 and min. 8) larvae per palm, respectively. As expected, presumed healthy palms were pest-free.

DISCUSSION

As expected, hormone-related defense pathways in *P. canariensis* were differentially affected by mechanical wounding (pruning) and infestation. Seven days after mechanical injury, palms showed increased concentrations of compounds related to the oxylipin and ABA defensive pathways. These two pathways are known to exhibit a positive cross-talk which usually antagonizes the onset of SA-dependent defenses (Flors et al*,* 2008; Glazebrook, 2005; Del Pozo, López-Matas, Ramírez-Parra, & Gutiérrez, 2005; Pieterse, Leon-Reyes, Vander Ent, Van Wees, 2009). Indeed, SA did not change in mechanically damaged palms relative to control and SAG even decreased in those palms. ABA and JA pathways have been typically associated to plant defense against wounding (Robert-Seilaniantz, Grant, & Jones, 2011) but also against insects with chewing mouthparts (Erb et al, 2012) as *R. ferrugineus*. Therefore, it is not surprising that 14 days after infestation, infested palms also showed enhanced levels of substances related to the oxylipin and ABA pathways. However, in this case the well-documented negative cross-talk of JA with SA-defensive pathways (Thaler*,* Humphrey & Whiteman, 2012) found by Giovino et al (2015) in presumably heavily infested *P. canariensis* was not observed. Indeed *R. ferrugineus* infested palms in our assay showed high increases of SA and SAG compared to control and mechanically-injured plants. Whether these differences should be attributed to the age of the palms or to the actual infestation density (15-20 years and unknown, respectively, for palms used by Giovino et al, 2015) deserves further investigations. Interestingly, the apparent deregulation of the negative SA-JA crosstalk documented in *P. canariensis* for *R. ferrugineus* has been reported in other cases of herbivory (Kant, Ament, Sabelis, Haring, & Schuurink, 2004; Kawazu et al, 2012; Agut et al, 2014).

Table 1. Concentrations (mean ± SE, ng/mg DW) of different plant hormones and secondary metabolites in control (C, time 0), mechanically injured (MI: control palms 7 days later after pruning), and *R. ferrugineus*-infested palms 7 and 14 days after infestation (7 dpi RPW and 14 dpi RPW, respectively). C and MI values were compared with a dependent t-test for paired samples (df = 11 in all cases, see table 2). Each of these values was subsequently compared with 7 and 14 dpi RPW with 1-way ANOVA (df = 2, 35 in all cases, see Table 2) and Tukey post-hoc test was used for mean separation.

	C	MI	C versus MI	7 dpi RPW	14 dpi RPW	RPW versus	
						C	MI
SA	385.02 ± 79.06	297.30 ± 48.43	C = MI	1608.91 ± 167.94	2471.83 ± 462.72	C < 7 = 14 dpi	MI < 7 = 14 dpi
IAA	166.22 ± 27.93	272.15 ± 77.99	C = MI	2308.98 ± 238.60	1115.14 ± 175.72	C < 14 < 7 dpi	MI < 14 < 7 dpi
CA (µg/ g DW)	100.61 ± 33.84	74.53 ± 17.31	C = MI	1009.70 ± 281.06	1016.34 ± 1917.01	C < 7 = 14 dpi	MI < 7 = 14 dpi
FA (µg/ g DW)	3.64 ± 1.00	7.44 ± 1.73	C = MI	16.09 ± 2.05	9.40 ± 1.02	C < 14 < 7 dpi	MI = 14 < 7 dpi
ChA	229.28 ± 111.69	221.48 ± 213.28	C = MI	464.05 ± 237.15	251.82 ± 263.26	C = 7 = 14 dpi	MI = 7 = 14 dpi
Ci (µg/ g DW)	1.66 ± 0.58	1.98 ± 0.47	C = MI	UD	UD	C > 7 = 14 dpi	MI > 7 = 14 dpi
ABA	221.92 ± 57.56	334.61 ± 52.41	C < MI	927.22 ± 132.93	984.36 ± 112.10	C < 7 = 14 dpi	MI < 7 = 14 dpi
ABAGlu	225.41 ± 57.87	314.14 ± 53.31	C < MI	992.63 ± 123.53	1048.06 ± 106.38	C < 7 = 14 dpi	MI < 7 = 14 dpi
SAG	297.33 ± 80.49	79.23 ± 36.91	C > MI	1883.00 ± 312.80	6008.52 ± 898.98	C = 7 > 14 dpi	MI = 7 > 14 dpi
JA	296.81 ± 90.04	80.58 ± 8.52	C > MI	1047.10 ± 326.27	717.18 ± 113.37	C ≤ 14 ≤ 7 dpi	MI ≤ 14 ≤ 7 dpi
JA-Ile	UD	1571.14 ± 363.94	C < MI	9.66 ± 6.84	1576.02 ± 363.21	7 < 14 dpi*	MI = 14 > 7 dpi

SA: Salicylic Acid; IAA: Indole-3-acetic Acid; CA: Caffeic Acid; FA; Ferulic Acid; ChA: Chlorogenic Acid; CiA: Cinnamic Aid; ABA: Abscisic Acid; ABAGlu: ABA-glucoside; SAG: Gulcosyl-salicylate;JA: Jasmonic Acid; JA-Ile: JA-isoleucine; UD: undetectable; NA: not applicable.

*Means compared using a t-test (df = 11).

Table 2. Results of the 1-way ANOVA (df = 2, 35 in all cases used to compare different phytophormone concentrations in control (C) and mechanically injured palms 7 days after injury (MI) with 7 and 14 dpi *R. ferrugineus*-infested palms (7 dpi RPW and 14 dpi RPW, respectively) (see table 1). C and MI values were compared with a dependent t-test for paired samples (df = 11 in all cases) and each of these values was subsequently compared with 7 and 14 dpi RPW with 1-way, see Table 2).

	C palms compared with MI	RPW-injured palms compared (*F*; *P*) with	
	(*t*; *P*)	C	MI
SA	1.082; 0.302	14.44; <0.001	16.02; <0.001
IAA	-1.278; 0.227	42.56; <0.001	36.3; <0.001
CA	0.671; 0.516	7.76; 0.002	8.28; 0.001
FA	-1.984; 0.073	20.12; <0.001	7.91; 0.002
ChA	0.030; 0.976	0.29; 0.7501	0.25; 0.7803
Ci	-0.400; 0.697	NA	NA
ABA	-3.819; 0.003	17.59; <0.001	12.84; <0.001
ABAGlu	-2.857; 0.016	23.12; <0.001	18.58; <0.001
SAG	2.515; 0.029	31.18; <0.001	33.32; <0.001
JA	2.349; 0.039	3.59; 0.039	6.62; 0.004
JA-Ile	NA	20.28; <0.001*	10.12; <0.001

SA: Salicylic Acid; IAA: Indole-3-acetic Acid; CA: Caffeic Acid; FA; Ferulic Acid; ChA: Chlorogenic Acid; CiA: Cinnamic Aid; ABA: Abscisic Acid; ABAGlu: ABA-glucoside; SAG: Gulcosyl-salicylate;JA: Jasmonic Acid; JA-Ile: JA-isoleucine; UD: undetectable; NA: not applicable.

* Means were compared with a t-test (df = 11).

Additionally, an increase of auxins and, remarkably, phenolic compounds, as precursors of phenylpropanoid phytoalexins, was observed in infested palms and this was not the case of mechanically wounded palms. These results are in agreement with Giovino et al (2015), who documented the upregulation of phenylalanine metabolism and phenylpropanodiol biosynthesis genes in *P. canariensis* from the middle stage of infestation. Phenolic compounds are known to possess insecticidal properties (Lattanzio, Lattanzio, & Cardinali, 2006) as they are involved in the formation of physical barriers as components of lignin and reducing the palatability of the plant (Lattanzio et al, 2006; Burghardt, Proksch, & Fiedler, 2001). Our results, which show that these substances can be detected as early as 7 days after infestation, demonstrate that *P. canariensis* activates defense responses against *R. ferrugineus* soon after oviposition/feeding damage starts, much earlier than reported by Giovino et al (2015). In agreement with these authors, the response of *P. canariensis* against *R. ferrugineus* cannot counterbalance the attack. Nevertheless, this response may offer a unique signature to detect early infestations. Because the observed changes appeared soon after exposure to adult ovipositing females, substances as caffeic acid and JA could be further exploited as early warning signs of infestation and provide an extremely useful tool for the management of this deadly pest.

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